

THE INDUCTIVE ROLE OF THE YOLK EPITHELIUM IN THE DEVELOPMENT OF THE SQUID, *LOLIGO PEALII* (LESUEUR)^{1, 2}

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While a fairly large body of information exists on the descriptive aspects of the embryology of the cephalopods, relatively little experimental work has been done on these animals. These embryos seem superficially to be ideally suited to embryological analysis since they are large, abundant, and are relatively easy to obtain (Arnold, 1962). One of the major difficulties has been the inability to work with embryos outside of the chorion. Ranzi (1931) tried to raise *Sepia officinalis* in sea water but had little success. He was able to isolate various organs and parts of the embryos of *Sepia* by operating through the chorion, and concluded that isolated organs and tissues were capable of self-differentiation. This technique severely limited operative procedures, and he was forced to use rather late stages. (Naef stage XII = Arnold stage 26) in which most of the organ primordia were rather well formed.

In the summer of 1961 it was possible to devise culture techniques by which the dechorionated embryos would survive and develop normally. This has allowed some experimental analysis of young embryos of the common Atlantic coast squid, *Loligo pealii*.

MATERIALS AND METHODS

Since the techniques used in this study have not been fully described, it is necessary to give a detailed account of the procedures used. Most of the experiments were performed *in vitro* in a culture medium made of three basic components: whole adult squid blood, sterile sea water, and an antibiotic stock solution. The blood was drawn under sterile conditions from the vena cava of the adults and stored frozen in 3-ml. glass tubes. Before use it was thawed and diluted with an equal volume of sterile sea water. To this was added about 1% of an antibiotic stock solution composed of 0.5% streptomycin, 0.05% phenol red, and 50,000 units of potassium penicillin G in double-distilled water which had been saturated with sulfadiazine. The embryos were cultured in glass vessels made expressly for this purpose. Each of these vessels had a volume of about 0.1 ml. and had two small depressions in the bottom for holding the embryos. This culture vessel was placed

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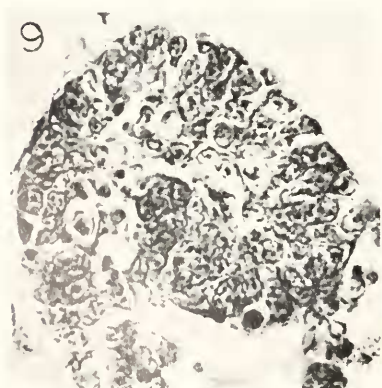
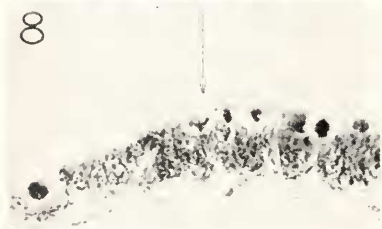
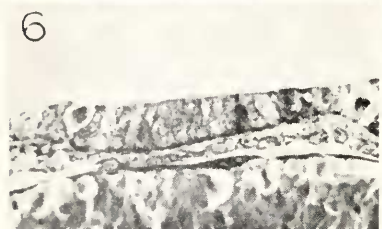
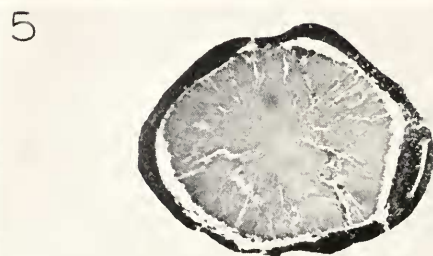
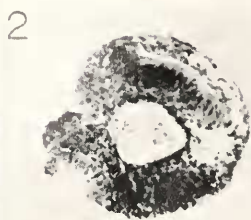
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in a covered preparation dish to which a few drops of sterile sea water had been added to saturate the atmosphere and maintain osmotic equilibrium with the medium in the vessel.

The embryos were prepared for culture by stripping off the outer tunics of the egg string with the fingers, cutting up the denuded string and transferring individual embryos in their chorions through two changes of sterile sea water. The chorion was then torn open and the embryo washed twice in sterile sea water. The operative procedures described below were performed and the embryo was then transferred to the freshly prepared medium. The embryos were incubated at 18°C . ($\pm 0.5^{\circ}\text{C}$.) and examined twice daily. The medium was changed every second day when the experiments exceeded this length of time.

Under these conditions the embryos appeared to develop completely normally when compared with control embryos still in their chorions and in normal sea water. The cultured embryos seemed to develop slightly faster than the controls, possibly because of greater availability of oxygen due to the lack of the chorion. Some embryos were maintained for 178 hours in culture with no apparent adverse effects. If the medium was not changed at least every three days, development would slow down. This effect was reversed by the addition of fresh medium.

Parts of the stage 16 and 17 embryos (Arnold, 1965) were removed with stainless steel needles before culturing. At these stages, the cephalopod embryo consists essentially of an outer layer of cells (future body of the embryo), a layer of greatly flattened cells (yolk epithelium), and a central mass of yolk. The various organ primordia could be identified by the thickened nature of the outer layer of cells (columnar *vs.* cuboidal cells) and the asymmetrical nature of the egg. Most of the operations involved removal of the parts of the eye, but the same confirming experiments were also done on the otocyst, arms and funnel folds. Three different types of operations were performed. The first involved the removal of the whole eye primordium, including some of the underlying yolk. This isolate would round up and the yolk would become incorporated within the center. These isolates survived quite well in the culture medium and underwent considerable differentiation. The second operation separated the outer layer of cells from the yolk epithelium. This was accomplished by gently rubbing the cells with the blunt edge of a needle until the cells became loosened and somewhat sticky. The outer layer of cells could then be caught on the point of a needle and pulled off in a sheet. A second needle was used to cut the sheet of cells well beyond the organ primordium. With practice, about one-fourth to one-third of the total surface of the outer cells could be removed and the embryo transferred to culture medium with only a few minutes exposure to sterile sea water. Sections of unstripped and stripped embryos are shown in Figures 1 and 4. In a few cases the eye anlage was removed by the technique described below for dissociating cells. A large number of embryos was examined and appropriate ones were selected. In the third operation the outer layer of cells was stripped and a small portion of the yolk epithelium removed. This was rather hard to accomplish since the embryos tended to lose yolk from the wound, which mechanically inhibited wound healing. However, enough cases were successful so interpretable results were obtained. In a few cases masses of cells were grafted onto the freshly stripped yolk epithelium. These cells were obtained by cutting up an egg string and shaking it in sea water adjusted to pH 5 with 1 N HCl



FIGURES 1-10.

until the egg-jelly dissolved. Once the egg-jelly was completely dissolved and the embryos inside their chorion were completely free, the embryos were shaken violently for about seven to ten minutes. The outer layer of cells was dissociated from the embryos, and usually single cells or small groups of cells resulted. The embryos were immediately examined, and those which appeared to be well dissociated were selected. At stage 17 the dissociated cells would reaggregate and form small solid clumps in about 15 minutes. These small clumps would fuse until about seven to fifteen aggregates remained in each chorion. Although these clumps would remain alive for extended periods, no organ or tissue differentiation was ever observed. Newly formed aggregates (one to two hours after dissociation) were stained in a solution of neutral red (0.01%) and grafted onto the freshly stripped yolk epithelium. The aggregates stuck quite readily and tended to flatten out on the surface of the embryo. The aggregates remained visible and were easily distinguished from the cells of the host embryo by their dye content.

RESULTS

When the whole eye primordium was removed, together with some of the surrounding tissue and underlying yolk, the resultant isolate rounded up with the yolk on the inside. These isolates differentiated quite normally and complete organs were formed (Figs. 2 and 3). The wound on the donor embryo lost a portion of yolk, but eventually the edges of the wound closed, development continued, and the unoperated organs differentiated normally. There was no evidence of any regeneration or replacement of the organs removed (Fig. 5). A total of 25 operations was performed on stage 17, and 13 operations on stage 16 embryos. In all cases the results of these operations were the same. The possibility of inhibition of development due to operative trauma was checked by a series of sham operations in which either less than the whole eye primordium was removed or a few cuts were made in the surface of the embryo. In these cases there was loss of yolk at the site of the wound but after healing normal development ensued.

Removal of the outer layer of cells resulted in a different pattern of development. The isolated cells usually formed a small clump that showed no further differentiation and lost the characteristics formerly possessed. These small clumps of cells did not survive well, possibly due to the trauma of surgery. Of those that

FIGURE 1. Section of the eye region of a stage 17 embryo. Note the yolk epithelium and the columnar nature of the outer layer of cells. Epon 812, Azure II-methylene blue; 660 $\times$.

FIGURE 2. Isolated organs of the embryo shown in Figure 5. Both the outer layer of cells and the yolk epithelium were isolated; *ca.* 190 $\times$.

FIGURE 3. Lower section of the same isolate as in Figure 2. In culture 45 hours.

FIGURE 4. Section of the yolk epithelium after removal of the outer layer of cells. One yolk epithelium cell has rounded up abnormally above the rest of the yolk epithelium; 660 $\times$.

FIGURE 5. Donor embryo for Figures 2 and 3. Note lack of organs on one side.

FIGURE 6. Regenerated otocyst after removal of the outer layer of cells; 900 $\times$.

FIGURE 7. Control eye for Figure 10; 725 $\times$.

FIGURE 8. Section through the retina of a stripped embryo onto which dissociated-reaggregated cells were grafted. In culture 40 hours; 1000 $\times$.

FIGURE 9. Dissociated-reaggregated cells after 55 hours. Note the lack of any tissue differentiation; 1000 $\times$.

FIGURE 10. Regenerated eye after removal of only the outer layer of cells. Compare with Figure 7.

did survive, no evidence of differentiation could be detected. The wound produced by this operation was closed by migration of the surrounding outer layer of cells over the denuded yolk epithelium. In covering the yolk epithelium the cells would cut off any small blebs of yolk that occasionally resulted from small punctures accidentally produced in the yolk epithelium during the operation. That these cells of the outer layer actually migrated over the wound rather than grew there was demonstrated by the speed at which the covering took place (one to two hours) as well as by a series of careful observations during wound closure. A total of 21 stage 17 and 10 stage 16 embryos was operated on in this fashion and all were cultured for at least 45 hours. In all of these cases the cells which migrated over the stripped yolk epithelium differentiated with the structures that would have normally differentiated in that site. In some cases (8) one-third of the total outer layer of cells was removed, yet the resultant embryos had all of their organs when examined and sectioned after approximately 48 hours in culture (Figs. 7 and 10). There seemed to be a slight retardation of development of the replaced organs but otherwise the course of development corresponded exactly to that of the unoperated side of the embryo. The eye, otocyst, arms and funnel folds could be thus stripped off and replaced by the cells normally destined to form other organs (*e.g.*, Fig. 6).

Since the above results would implicate the yolk epithelium as possibly having a causal role in the differentiation of the overlying cells, two other experiments were attempted to test this hypothesis. The first of these involved stripping off the outer layer of cells of stage 17 embryos and removing a small portion of the yolk epithelium. This was not easily accomplished because the yolk epithelium was easily torn. However, in two cases the yolk epithelium in the future region of the otocyst was successfully removed and development was carefully followed. In this case closure of the wound proceeded as before and differentiation of the organs followed but the embryo lacked the otocyst on the operated side. The second test involved grafting dissociated-reaggregated cells onto the freshly denuded yolk epithelium. Normally these aggregated cells survived in the culture medium for extended periods and gradually died and disintegrated. In no case did any of the aggregates resulting from well dissociated embryos ever show any interpretable differentiation (Fig. 9). When these cells were grafted onto the denuded yolk epithelium, they stuck quite readily and spread out slightly. The position and extent of the grafted cells could be easily ascertained by the dye they contained. When sectioned it appeared that those cells in contact with the yolk epithelium differentiated into rather normal-looking tissue which corresponded to the location on the embryo (Fig. 8). In the case of the primordial retina, the cells became columnar in appearance, underwent mitosis in the characteristic position, and appeared to be well incorporated in the host organ. In eight successful cases, parts of four grafts unquestionably became incorporated into the developing retina. In the remaining case the results were not as clearcut because of yolk leakage at the site of the graft.

The possibility of the yolk playing a significant specific causal role in the differentiation was eliminated by experimentally removing up to one-half of the yolk of the embryo. Despite this rather large loss in volume the embryos differentiated normally but were reduced in size, particularly in the yolk sac region. These results agree with those obtained by Okada (1927) on *Loligo bleekeri*.

DISCUSSION

The results reported have led the author to the conclusion that the cells of the outer layer are indeterminate in their fate unless influenced by the underlying yolk epithelium. This appears to fit the classic ideas of embryonic induction, with the yolk epithelium being the inductor and the outer layer of cells responding to its inductive influence. This can be stated with certainty for the eye and otocyst and as probable for some of the other organs (funnel folds, arms and gills). Organs other than these remain to be tested, but preliminary experiments indicate that their development is of the same general nature. It appears, therefore, that all of the organs of the embryonic body are laid out in inductive areas of the yolk epithelium and are fairly localized. This can be visualized as a "morphogenetic inductive map" in which developmental information is transferred to the highly labile or indifferent cells that overlie it. One of the rather unique features of this inductive map is its two-dimensional nature. Unlike many other systems in which induction occurs within a mass of heterogeneous tissue, the inductor in this case is present as a sheet. This system, therefore, should be more amenable to experimental analysis because the presumptive areas of the embryo can be rather easily localized and subjected to direct experimental analysis. Preliminary experiments with chemical treatment of the denuded yolk epithelium offer encouragement along these lines.

The problem of how to fit these results with the classical ideas of mosaic development of the molluscs is an open question. It is obvious that part of the embryo (the yolk epithelium) is rather rigidly fixed in its fate while the outer layer of cells is quite labile and subject to the inductive influences of the yolk epithelium. Just when and how this developmental information arises is still unknown but preliminary experiments indicate that the egg cortex of this embryo is also rather rigidly patterned and the morphogenetic patterns would then be referred ultimately to the ovary. Obviously, further work along these lines is indicated.

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SUMMARY

1. Techniques for the *in vitro* culture of dechorionated *Loligo pealii* embryos, involving the use of whole adult squid blood, sterile sea water, and antibiotics, have been devised. Essentially normal development will take place in this culture medium.

2. The embryos in stages 16 and 17 are composed essentially of three components: an outer layer of cells, an inner cellular yolk epithelium, and the central mass of yolk. When the outer layer of cells and the yolk epithelium are isolated together, normal histogenesis and development will occur. When the outer layer of cells is isolated by itself, no differentiation occurs. This leads to the conclusion that the yolk epithelium induces the outer layer of cells to differentiate. This conclusion was upheld by grafting and deletion experiments.

3. The role of the yolk epithelium, therefore, may be in acting as a "morphogenetic inductive map."

LITERATURE CITED

- ARNOLD, J. M., 1962. Mating behavior and social structure in *Loligo pealii*. *Biol. Bull.*, **123**: 53-57.
- ARNOLD, J. M., 1965. Normal embryonic stages of the squid, *Loligo pealii* (Lesueur). *Biol. Bull.*, **128**: 24-32.
- NAEF, A., 1923. Die Cephalopoden. Monographie 35. Fauna e Flora del Golfo di Napoli, Vol. 1.
- OKADA, Y., 1927. Versuche über die Wirkung der Dotterwegnahme am meroblastischen Ei. *Zool. Anz.*, **73**: 280-284.
- RANZI, S., 1931. Sviluppo di parti isolate di embrioni di Cefalopodi. *Pubb. Staz. Zool. Napoli*, **11**: 104-146.